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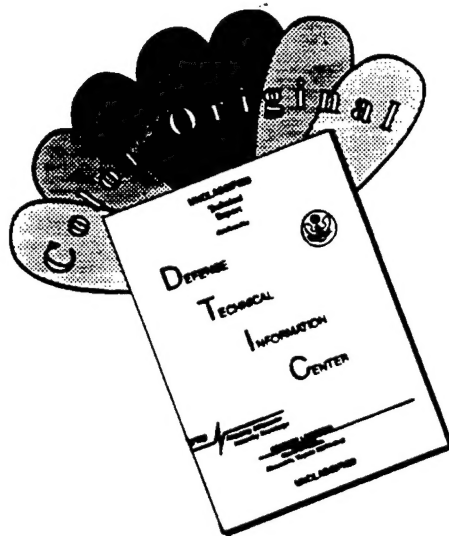
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Stromelysin-3, a new member of the matrix metalloproteinase family, is specifically expressed in stromal cells surrounding invasive breast carcinoma cells where it undergoes processing to its active form by a proprotein convertase-dependent pathway. We now report that stromelysin-3 does not exert direct matrix-degrading activities or alter the expression of an invasive phenotype *in vitro*. However, following targeted expression of the ST-3 transgene to the lactating mammary gland *in vivo*, a premature involution program appear to be initiated. Given the fact that matrix remodeling programs induced by breast cancer cells appears similar to those observed during mammary gland involution, these data suggest that ST-3 may play an important, but indirect, role in regulating tissue integrity *in vivo*. Furthermore, we demonstrate that the proprotein convertase-dependent activation mechanism used for ST-3 processing can be extended to a second breast cancer-associated matrix metalloproteinase, the membrane-type matrix metalloproteinase. Together, these data indicate that proprotein convertases may regulate a series of matrix metalloproteinases whose expression can exert complex effects on matrix remodeling programs associated with carcinoma invasion and metastasis *in vivo*.

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I.   INTRODUCTION

Current evidence suggests that breast carcinoma cells invade local tissues and metastasize by either expressing, or inducing the expression of, proteolytic enzymes that degrade structural barriers established by the extracellular matrix (ECM)[1-3]. Although the identity of the specific proteinases that lend cancer cells their invasive potential remains the subject of conjecture, attention has recently focused on the matrix-degrading metalloproteinases (MMPs) - a family of at least 14 zinc-dependent proteolytic enzymes whose overlapping substrate specificities include all of the major components of the ECM (since the last progress report, three new MMPs have been characterized, MT-MMP-2, MT-MMP-3 and MT-MMP-4; see below)[1-5]. Consistent with their presumed role in tumor progression, *in situ* analyses of a variety of cancerous tissues have confirmed heightened levels of expression of one or more MMPs in tumor and/or surrounding stromal tissues[6-8]. Furthermore, a range of *in vitro* as well as *in vivo* studies have demonstrated that invasion and metastasis can be affected by altering MMP activity[9-12]. Given these findings, efforts have intensified to identify those MMPs that might be used as diagnostic indicators or potential targets for pharmacologic intervention in breast as well as other cancers.

Until recently, attempts to implicate specific MMPs in breast cancer progression were based on the assumption that all of the major proteinases belonging to this gene family had been identified and characterized[2]. Unexpectedly however, differential screens of breast cancer cDNA libraries led to the tentative identification of a new member of the MMP family, termed stromelysin-3, on the basis of its apparent homology to stromelysin-1 and -2 (two other members of this gene family that had been previously characterized)[13]. The expressed gene product was predicted to encode a 488-residue protein containing i) a candidate leader sequence, ii) a highly conserved PRCGXPD motif believed to maintain the latency of MMP zymogens, iii) a zinc-binding catalytic motif and iv) a carboxyl-terminal domain with sequence homology to the heme-binding protein, hemopexin and the ECM molecule, vitronectin[13]. Interestingly, in all invasive breast carcinomas examined thus far, ST-3 was not a product of the neoplastic cells themselves, but rather the surrounding stromal cells[6,12,13].

Given the structural similarity between ST-3 and other members of the MMP family, it was postulated that the enzyme would be secreted as a zymogen whose extracellular activation at the tumor-stroma interface would follow the destabilization of the ligand formed between the Cys in the PRCGVPD domain and the Zn^{+2} in the catalytic domain[6,12,13]. In a scheme analogous to that established for the other MMPs, ST-3 could then under autoproteolytic processing to a mature, active form which presumably would catalyze the degradation of critical ECM components localized in either the basement membrane or stroma[4,5]. However, despite the structural similarities that exist between ST-3 and other MMP members, additional studies indicate that i) the primary sequence of ST-3 is distinct from all other members of the MMP family and ii) the assumed role of ST-3 in ECM remodeling cannot be readily confirmed[4,14]. First, comparisons of the catalytic domains of the MMPs suggest that ST-3 belongs in a new subgroup relative to all other members of this gene family and that its closest homology lies with the bacterial metalloproteinases[4]. Consistent with the contention that ST-3 is a structurally distinct entity in the MMP family, the human ST-3 gene has been localized to the long arm of chromosome 22, a position which differs from those of all other MMP genes[4,12]. Second, although the expression of the human ST-3 protein has not yet been reported, the homologous mouse enzyme (~80% homology identity at the amino acid level) could only be isolated in a truncated form that expressed weak proteolytic activity[14]. Indeed, these results led Murphy and colleagues to conclude that "the evidence that mature full length stromelysin-3 is a metalloproteinase could not be substantiated and the precise role of this protein *in vivo* remains to be elucidated."[14] Taken together, these findings

indicated that despite the provocative correlative link established between ST-3 expression and breast cancer progression, the role of this proteinase in invasive events remains undefined. In this proposal, we sought to use a series of *in vitro* as well as *in vivo* biochemical and molecular approaches to i)purify and characterize ST-3 expressed by stably-transfected mammalian cells, ii) determine the mechanism by which the ST-3 zymogen is activated and regulated, iii) determine the ability of stromelysin-3 to regulate the invasive potential of breast carcinoma cells *in vitro* and iv) characterize the role of ST-3 in regulating invasive potential *in vivo* in a transgenic mouse model. During the first year of the proposal, the first two major goals were completed. Since that time, efforts have begun to focus on characterizing the role of ST-3 in regulating the invasive phenotype of recipient cells and in generating a transgenic model.

II. BODY

During the previous granting period we demonstrated that the human ST-3 zymogen is processed to an active proteinase following intracellular processing by the proprotein convertase, furin[29]. In turn, active ST-3 was purified to homogeneity and shown to cleave and inactivate members of the serine proteinase family[30]. Interestingly, however, the enzyme displayed a highly restricted substrate specificity that did not extend to well-characterized extracellular matrix (ECM) components (i.e., in contradistinction to almost all other MMPs that express strong matrix-degrading activities, no ECM component could be identified as a ST-3 substrate). Nonetheless, these results do not allow one to conclude that more subtle effects might be exerted on the ECM *in vitro* or *in vivo*. To this end, we have i) examined the ability of ST-3 to affect basement membrane assembly and cell invasion and ii) generated and begun characterizing a line of transgenic mice that overexpress ST-3 in breast tissues. Finally, because our studies on *in vitro* analyses of ST-3-ECM interactions were negative (thus confirming our suspicion that ST-3 does not directly degrade the ECM), we iii) initiated efforts to determine whether the activation process identified for ST-3 might be extended to a new breast cancer-associated MMP, the membrane-type MMP-1 (MT-MMP-1)[31].

ST-3-DEPENDENT REGULATION OF BASEMENT MEMBRANE ASSEMBLY/DISASSEMBLY AND THE INVASIVE PHENOTYPE

a. Assembly of basement membrane model. In the original proposal, studies were outlined to assess invasion in two models, i.e., Matrigel (an extract of the ECM synthesized by the EHS tumor *in vivo*) or an *in vivo*-like basement membrane deposited by an immortalized epithelial cell line (MDCK strain II). However, because more recent studies indicate that the Matrigel model may be flawed (i.e., Matrigel-coated filters contain holes[32]), we instead focused efforts on the MDCK system. To generate an epithelial cell-derived basement membrane, a strain II MDCK cell clone was isolated which will deposit an intact basal lamina when cultured atop a type I collagen gel for 3 weeks in a Transwell construct (Figure 1, panel A; see Appendix)[33,34]. Following a gentle detergent lysis, the denuded, but intact, basement membrane can be readily observed by either transmission or scanning EM (panels B and D) and easily discerned from the underlying type I collagen (panel C). The structural integrity of the basement membrane was confirmed by assessing the inability of an ~250 Å-sized colloid to diffuse across the barrier into the underlying collagen as described in earlier studies from our laboratory[35]. Finally, in a manner consistent with earlier reports indicating that MDCK cells synthesize all of the major basement membrane components[33], immunohistochemical analyses have confirmed the presence of type IV collagen, laminin and heparin sulfate proteoglycan in the basement membrane (data not shown).

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b. Basement membrane assembly by ST-3-transfected MDCK cells. To first determine whether ST-3 might affect basement membrane, we initiated efforts to stably transfect the MDCK cells with human ST-3 with the intent of examining the ability of these cells to successfully deposit and maintain an intact basement membrane. To generate stable MDCK cell lines that produce human ST-3, an ST-3 expression vector was constructed by cloning the ST-3 cDNA into the Hind III-XhoI site of pREP9 under the control of RSV-LTR[30]. The pREP9-ST-3 plasmid was then transfected into MDCK cells by the calcium phosphate co-precipitation method[30]. The MDCK cells were grown in DMEM containing 10% FCS, glutamine and antibiotics as described[30]. After 24 h, the cells were selected with G418 and clonal lines maintained in selection media. Positive clones were then screened by Western blotting (Figure 2). As shown, multiple MDCK clones expressed high levels of the ST-3 protein, which as predicted by our earlier studies[29,30] was secreted as the fully active enzyme (in some of the clones, a small portion of the ST-3 escaped processing and was secreted as the zymogen). In addition, all of the ST-3 expressing clones generated ST-3 fragments as a consequence of the previously described ability of active enzyme to undergo autocatalytic processing[30].

To determine the ability of the MDCK stable clones to generate an intact basement membrane while expressing active ST-3, the cells were grown atop type I collagen gels as described above. Interestingly, the ST-3 overexpressing clones generated basement membranes in a fashion identical to that observed in control MDCK cell lines (Figure 3, panels A and B). In data not shown, cell-free supernatants recovered from the ST-3-transfected clones contained high levels of the active enzyme (as assessed by its ability to cleave β-casein[30]). Thus, active ST-3 did not exert a discernible effort in the assembly of the basement membrane during a 3-week culture period.

c. Effect of ST-3 overexpression on invasive behavior. To determine the effect of ST-3 expression on invasive activity, we took advantage of our recent observation that MDCK cells can be induced to invade across their own basement membrane and undergo a tubulogenic program following stimulation with the mesenchymal-derived growth factor, scatter factor[36] (unpublished observation). Because scatter factor expression is increased in breast cancer[36] and scatter factor-stimulated MDCK cells express a panel of gene products similar to those described in invasive breast carcinomas (i.e., collagenase-3, TIMP-3, and MT-MMP-1; unpublished observation), we compared the ability of control versus the ST-3-transfectants to express an invasive phenotype. As shown in Figure 3, when ST-3-transfected cells were stimulated with scatter factor they began to cross the membrane and to generate tubules (panels C and D). However, the rate at which these events occurred was no different from the control MDCK cells (data not shown). Hence, ST-3 neither affected the basement membrane assembly program nor the invasion program expressed by epithelial cells. Interestingly, these results are consistent with the recent observation by Noel et al[37] that ST-3-transfected MCF-7 cells do not display a heightened invasive or metastatic phenotype *in vivo*.

GENERATION OF ST-3-OVEREXPRESSING TRANSGENIC MICE

In order to assess the impact of ST-3 overexpression on tumor progression *in vivo*, initial efforts focused on an attempt to target transgene expression to the stroma (i.e., ST-3 is "normally" expressed in the stroma surrounding invasive carcinomas[38]) with the intent of using the transgenic mice as recipients for mammary cell tumor lines which would be injected into mammary fat pads. To this end, a hybrid transcriptional unit comprising the long terminal repeat of RSV and human ST-3 cDNA was microinjected into 300 mouse embryos and implanted into 10 mice in

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collaboration with the Transgenic Core Facility at the University of Michigan. (RSV was chosen since earlier studies suggested that i) expression appears limited to cells or tissues of mesodermal origin and ii) fusion constructs using RSV LTR are not expressed at embryonic stages of development thus obviating potentially disruptive alterations that might occur during embryogenesis[39].) Thirty-one mice were born and screened for transgene integration by Southern blotting using DNA isolated from tails[40]. As shown in Figure 4, four positive mice were identified and subsequently analyzed for germline transmission by breeding and Southern blotting. As a result (data not shown), we demonstrated that the transgene was integrated into the germline of each of the founders.

To determine the tissue expression pattern of the ST-3 transgene, transgenic mice were produced by breeding each founder and transgene-positive animals identified by PCR of tail-derived DNA[40]. Positive mice (10 mice as a group) were dissected to obtain various organs and tissues including: brain, skin, liver, lung, kidney, tail, bone, mammary gland, intestine and muscle. Fresh tissue and organs were immediately frozen in liquid nitrogen and homogenized in guanidine isothiocyanide solution. Total RNA was isolated by phenol/chloroform extraction and precipitated with 100% ice-cold ethanol as described[40]. RNA samples (10 µg each) were electrophoresed on agarose gel, transferred to nitrocellulose membranes and hybridized with [^{32}P]-labeled human ST-3 probe. Following extensive analyses, ST-3 RNA could not be detected in any of the tissues examined. The screening experiments were repeated, but identical results were obtained.

The inability to detect transgene expression suggested that in viable founders, the transgene was preferentially localized in transcriptionally silent regions of the genome. Given that transgene expression may have been deleterious to normal development (despite reports of limited expression of the RSV promoter *in utero*[39]), we alternatively opted to place ST-3 expression under the control of regulatory elements of the mammary epithelial cell-specific, pregnancy responsive whey-acidic protein (WAP) gene[40,41]. Because ST-3 is normally expressed during mammary gland involution (a state that closely parallels many of the matrix-remodeling events observed in carcinomas[42,42a]), our rationale was to inappropriately express the ST-3 transgene during midpregnancy and lactation in order to observe the effect exerted on the well-characterized mammary gland phenotype. To this end, transgenic mice were generated using a chimeric recombinant DNA composed of the WAP promoter and the entire ST-3 coding region. The WAP promoter was a 2.6-kb DNA corresponding to the 5' end of the WAP gene extending into the first exon just 5' of the first AUG as described[41]. The WAP-ST-3 construct was then injected into 300 embryos and implanted into 10 mice. Following a screening and breeding plan similar to that described above for the RSV-ST-3 construct, eight positive mice were obtained (Figure 5) with the transgene integrated into the founder germlines. When tissues from transgenic mice were examined by RT-PCR (not shown) and Northern blot analysis for tissue-specific expression of the transgene, ST-3 expression was limited to the mammary glands of pregnant and lactating female mice (with low levels in the brain; Figure 6). While we have only begun to characterize the phenotype of the WAP-ST-3 transgenics, preliminary studies indicate that ST-3 may trigger a premature involution program. As shown in Figure 7, whereas normal mammary glands undergo intensive lobuloalveolar development during pregnancy and lactation, the lactating transgenic gland is markedly smaller and appears to have initiated a premature involution program. While cross-sections of the transgenic glands show numerous collapsed alveoli (see Figure 7), some functional ducts remain. This mosaic pattern of premature involution is consistent with the previously described heterogeneous expression of transgenes in mammary glands[43]. If these data are substantiated in our founder lines, the results would provide the first proof that ST-3 participates directly in the mammary involution program.

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ACTIVATION OF THE MT-MMP-1 ZYMOGEN

While attempting to delineate the mechanisms by which ST-3 regulates tumor progression, we were intrigued by the demonstration that stromal cells surrounding carcinoma cells also expressed an additional new member of the MMP family, i.e., MT-MMP-1[31], with structural similarities to ST-3. Unlike ST-3, however, MT-MMP-1 is not a secretory protein, but is instead expressed as a membrane-anchored protein[31]. Consequently, attempts to characterize MT-MMP-1 activity were hindered by technical problems associated with isolating and purifying a membrane-associated molecule. However, we noted that with the exception of an extended C-terminal domain (which includes a hydrophobic transmembrane domain and a short cytosolic tail), the modular organization of MT-MMP-1 was identical to that of the secreted MMPs[44]. Hence, soluble mutants of MT-MMP-1 were generated and expressed[44]. Interestingly, these MT-MMP-1 mutants (denoted as ΔMT-MMP) were recovered as fully mature, processed proteinases rather than as zymogens. Thus, these results were highly reminiscent of the novel activation pathway that we previously described for ST-3. In the latter case, the stromelysin-3 zymogen was processed directly to its enzymically active form by an obligate intracellular proteolytic event that occurred within the constituitive secretory pathway[29]. Endoproteolytic processing of stromelysin-3 was subsequently shown to be regulated by an unusual 10-amino acid insert that is sandwiched between the pro- and catalytic-domains of the zymogen and encrypted with an Arg-X-Arg-X-Lys-Arg (RXRXKR; where R = Arg and K = Lys) motif. Recent studies have demonstrated that protein precursors that display a triad of basic residues arranged in an Arg-X-Lys/Arg-Arg array can be cleaved on the C-terminal side of the consensus sequence by members of a family of intracellular serine proteinases known as the proprotein convertases[29]. In the case of stromelysin-3, a single member of the convertase family (currently believed to contain at least 7 distinct enzymes), termed furin, was shown to play a pre-eminent role in the processing event[29]. In this manner, the basic motif was "read" by an intracellular processing proteinase concentrated in the *trans*-Golgi network that removed the 97-amino acid propeptide - thus generating active stromelysin-3 prior to its secretion. Because no other members of the MMP family contained a similar furin recognition motif, it was presumed that this processing pathway would specifically apply to stromelysin-3 alone. Interestingly, however, with the recent cloning of MT-MMP[31], it became clear that a second member of the MMP family contained a similar (but distinct RRKR insert) insert. [Indeed, we have completed characterizing the genomic organizations of stromelysin-3 and MT-MMP and have found - contrary to our prediction - that phylogenetic tree and chromosomal localization analyses indicate that stromelysin-3 and MT-MMP are not closely related and form separate branches within the MMP family tree; unpublished observation.] Given that the active form of ΔMT-MMP (as well as the active form of the wild-type MT-MMP) displays an N-terminus directly downstream of a motif similar to that found in stromelysin-3[44], we initiated studies to delineate the importance of this insert.

a) Alanine scanning mutagenesis of the ΔMT-MMP$_{II}$ RRKR motif. Currently, three members of the proprotein convertase family are known to recognize Arg-X-Lys/Arg-Arg motifs in the constituitive secretory pathway; furin, PACE4 and PC5/6[29,44]. Sequence rules established for precursor cleavage by furin/PACE4-like convertases (PC5/6 rules have not been extensively characterized) indicate varying roles for the basic residues at positions -1, -2, -4, -6 and possibly, -8 relative to the scissile bond (i.e., P^{-1}, P^{-2}, P^{-4}, P^{-6}, and P^{-8}, respectively)[45]. Thus, the amino acid sequence requirements for ΔMT-MMP processing in COS cells were compared in transient transfection assays where the Arg residues at P^{-4} or P^{-1} were substituted for Ala residues. As shown in the accompanying manuscript (see Appendix), each of these substitutions almost completely blocked processing. In contrast, a similar substitution at the less critical P^{+1} site did not affect ΔMT-MMP$_{II}$ processing. Although the role of basic residues at P^{-2}, P^{-3} and P^{-8} remain to be determined (and with the assumption that the insert substitutions did not significantly perturb the conformation of the [111]Arg-Tyr cleavage site), these results suggest that a proprotein convertase recognition motif is critical to the ΔMT-MMP$_{II}$ activation process.

b) Effect of the Pittsburgh variant of α_1*PI (*α_1*PI$_{Pitt}$) on ΔMT-MMP processing in COS cells.* COS cells are known to express only two members of the proprotein convertase family that recognize Arg-X-Lys/Arg-Arg motifs, i.e., furin and PACE4[29,44]. To determine which of these enzymes participate in ΔMT-MMP processing, COS cells were co-transfected with ΔMT-MMP and the Pittsburgh mutant of $\alpha_1$proteinase inhibitor, α_1PI$_{Pitt}$, a reactive site variant that inhibits furin (but not PACE4) activity *in situ*[29,44]. Under these conditions, α_1PI$_{Pitt}$ (but not α_1PI) completely blocked ΔMT-MMP processing. Significantly, purified pro-ΔMT-MMP was unable to process progelatinase A (a natural substrate for MT-MMP) confirming the importance of the mature form of the enzyme. However, when purified ΔMT-MMP was incubated with a mutant soluble form of furin (generated by deleting its TM domain; see ref. 29), the mature form of the proteinase was generated and proven effective as a progelatinase activator. These results indicate that pro-ΔMT-MMP can be effectively processed to its active form via a furin-dependent pathway in COS cells and that proprotein convertases play a broader role in MMP activation cascades in breast cancer than previously considered.

III. CONCLUSION

The research completed to date have allowed us to demonstrate that although human ST-3 may not directly regulate basement membrane assembly or the expression of an invasive phenotype, its *in vivo* expression may trigger a massive matrix remodeling program. These data provide the first insights into ST-3 function *in vivo* and strongly suggest that the proteinase may be generated in concert with an additional network of accessory factors. Furthermore, our results also indicate that a recently discovered new MMP, MT-MMP-1, is regulated by a proprotein convertase-dependent pathway similar, if not identical, to that described for ST-3. Because ST-3 as well as MT-MMP-1 are up-regulated in primary as well as metastatic breast cancer, our results indicate that pharmacologic interventions directed against MMPs or proprotein convertases could exert important therapeutic effects.

IV. REFERENCES

1. Laiho, M., Keski-Oja, J. Growth factors in the regulation of pericellular proteolysis: A review. Cancer Res. 49:2533-2553, 1989.
2. MacDonald, N.J.and Steeg, P.S. Molecular basis of tumour metastasis. In Cancer Surveys, The Molecular Pathology of Cancer, 16:175-199, 1993.
3. Liotta, L.A., Steeg, P.S., and Stetler-Stevenson, W.G. Cancer metastasis and angiogenesis: An imbalance of positive and negative regulation. Cell 64:327-336, 1991.
4. Matrisian, L.M. The matrix-degrading metalloproteinases. BioEssays 14:455-463, 1992.
5. Woessner, J.F. Jr. Matrix metalloproteinases and their inhibitors in connective tissue remodeling. FASEB J. 5:2145-2154, 1991.
6. Wolf, C., Rouyer, N., Lutz, Y., Adida, C., Loriot, M., Bellocq, J.P., Chambon, P. and Basset, P. Stromelysin 3 belongs to a subgroup of proteinases expressed in breast carcinoma fibroblastic cells and possibly implicated in tumor progression. Proc. Natl. Acad. Sci. USA 90:1843-1847, 1993.
7. Monteagudo, C., Merino, M.J., San-Juan, J., Liotta, L.A., Stetler-Stevenson, W.G. Immunohistochemical distribution of type IV collagenase in normal, benign, and malignant breast tissue. Am J Pathol 136:585-592, 1990.
8. Polette, M., Clavel, C., Cockett, M., de Bentzmann, S.G., Murphy, G.and Birembaut, P. Detection and localization of mRNAs encoding matrix metalloproteinases and their tissue inhibitor in human breast pathology. Invasion Metastasis 13:31-37, 1993.
9. Mignatti, P., Robbins, E., and Rifkin, D.B. Tumor invasion through the human amniotic membrane: requirement for a proteinase cascade. Cell 47:487-498, 1986.
10. DeClerck, Y.A., Perez, N., Shimada, H., Boone, T.C., Langley, K.E., and Taylor, S.M. Inhibition of invasion and metastasis in cells transfected with an inhibitor of metalloproteinases. Cancer Res. 52:701-708, 1992.
11. Powell, W.C., Knox, J.D., Navre, M., Grogan, T.M., Kittelson, J., Nagle, R.B., and Bowden, G.T. Expression of the metalloproteinase matrilysin in DU-145 cells increases their invasive potential in severe combined immunodeficient mice. Cancer Res. 53:417-422, 1993.
12. Basset, P., Wolf, C. and Chambon, P. Expression of the stromelysin-3 gene in fibroblastic cells of invasive carcinomas of the breast and other human tissues: a review. Breast Cancer Res. Treatment 24:185-193, 1993.
13. Basset, P., Bellocq, J.P., Wolf, C., Stoll, I., Hutin, P., Limacher, J.M., Podhajcer, O.L., Chenard, M.P., Rio, M.C., and Chambon, P. A novel metalloproteinase gene specifically expressed in stromal cells of breast carcinomas. Nature 348:699-704, 1990.
14. Murphy, G., Segain, J.P., O'Shea, M., Cockett, M., Ioannou, C., Lefebvre, O., Chambon, P., and Basset, P. The 28-kDa N-terminal domain of mouse stromelysin-3 has the general properties of a weak metalloproteinase. J. Biol. Chem. 268:15435-15441, 1993.
15. Guan, K.L., Dixon, J.E., Eukaryotic protein expressed in Escherichia coli: An improved thrombin cleavage and purification procedure of fusion proteins with Glutathione S-transferase. Anal. Biochem. 192:262-269,1991.
16. Yates, J.L., Warren, N., Sugden, B. Stable replication of plasmids derived from Epstein-Barr virus in various mammalian cells. Nature 313:812-815, 1985.
17. Graham, F., and van der Eb, G.A. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology 52:456-457, 1973.
18. Desrochers, P.E., Mookhtiar, K., VanWart, H.E., Hasty, K.A., and Weiss, S.J. Proteolytic inactivation of α_1-proteinase inhibitor and α_1-antichymotrypsin by oxidatively activated human neutrophil metalloproteinases. J. Biol. Chem. 267:5005-5012, 1992.
19. Sottrup-Jensen, L. α-Macroglobulins: Structure, shape, and mechanism of proteinase complex formation. J. Biol. Chem. 264:11539-11542, 1989.
20. Davies, B., Brown, P.D., East, N., Crimmin, M.J., and Balkwill, F.R. A synthetic matrix metalloproteinase inhibitor decreases tumor burden and prolongs survival of mice bearing human ovarian carcinoma xenografts. Cancer Res. 53:2087-2091, 1993.

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21. Nagase, H., Ogata, Y., Suzuki, K., Enghild, J.J., and Salvesen, G. Substrate specificities and activation mechanisms of matrix metalloproteinases. Biochem. Soc. Trans. 19:715-718, 1991.

22. Desrochers, P.E., Jeffrey, J.J., and Weiss, S.J. Interstitial collagenase (matrix metalloproteinase-1) expresses serpinase activity. J. Clin. Invest. 87:2258-2265, 1991.

23. Winyard, P.G., Zhang, Z., Chidwick, K., Blake, D.R., Carrell, R.W., and Murphy, G. Proteolytic inactivation of human α_1 antitrypsin by human stromelysin. FEBS Lett. 279:91-94, 1991.

24. Keski-Oja, J., Lohi, J., Tuuttila, A., Tryggvason, K., and Vartio, T. Proteolytic processing of the 72,000-Da type IV collagenase by urokinase plasminogen activator. Exp. Cell Res. 202:471-476, 1992.

25. Strongin, A.Y., Marmer, B.L., Grant, G.A., and Goldberg, G.I. Plasma membrane-dependent activation of the 72-kDa type IV collagenase is prevented by complex formation with TIMP-2. J. Biol. Chem. 268:14033-14039, 1993.

26. Steiner, D.F., Smeekens, S.P., Ohagi, S., and Chan, S.J. The new enzymology of precursor processing endoproteases. J. Biol. Chem. 267:23435-23438, 1992.

27. Barr, P. J. Mammalian subtilisins: The long-sought dibasic processing endoproteases. Cell 66:1-3, 1991.

28. Liu, Y-C., Kawagishi, M., Mikayama, T., Inagaki, Y., Takeuchi, T., and Ohashi, H. Processing of a fusion protein by endoprotease in COS-1 cells for secretion of mature peptide by using a chimeric expression vector. Proc. Natl. Acad. Sci. USA 90:8957-8961, 1993.

29. Pei, D. and Weiss, S. Furin-dependent intracellular activation of the human stromelysin-3 zymogen. Nature 375:244-247, 1995.

30. Pei, D., Majmudar, G. and Weiss, S.J. Hydrolytic inactivation of a breast carcinoma cell-derived serpin by human stromelysin-3. J. Biol. Chem. 269:25849-25855, 1994.

31. Sato, H., Takino, T., Okada, Y., Cao, J., Shinagawa, A., Yamamoto, E., and Seiki, M. A matrix metalloproteinase expressed on the surface of invasive tumour cells. Nature. 370:61-65, 1994.

32. Imamura, H., Takao, S., and Aikou, T. A modified invasion-3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltertrazolium bromide assay for quantitating tumor cell invasion. Cancer Res. 54:3620-3624, 1994.

33. Ecay, T.W., and Valentich, J.D. Basal lamina formation by epithelial cell lines correlates with laminin A chain synthesis and secretion. Exp. Cell Res. 203:32-38, 1992.

34. Patrone, L.M., Cook, J.R., Crute, B.E., and Van Buskirk, R.G. Differentiation of epithelial cells on microporous membranes. J. Tissue Culture Meth. 14:225-234, 1992.

35. Huber, A.R., and Weiss, S.J. Disruption of the subendothelial basement membrane during neutrophil diapedesis in an in vitro construct of a blood vessel wall. J. Clin. Invest. 83:1122-1136, 1989.

36. Rosen, E.M., Nigam, S.K,. and Goldberg, I.D. Scatter factor and the c-Met receptor: A paradigm for mesenchymal/epithelial interaction. J. Cell Biol. 237:1783-1787, 1994.

37. Noel, A.C., Lefebvre, O., Maquoi, E., VanHoorde, L., Chenard, M.P., Mareel, M., Foidart, J.M., Basset, P., and Rio, M.C. Stromelysin-3 expression promotes tumor take in nude mice. J. Clin. Invest. 97:1924-1930, 1996.

38. Wolf, C., Rouyer, N., Lutz, Y., Adida, C., Loriot, M., Bellocq, J.P., Chambon, P., and Basset, P. Stromelysin 3 belongs to a subgroup of proteinases expressed in breast carcinoma fibroblastic cells and possibly implicated in tumor progression. Proc. Natl. Acad. Sci. 90:1843-1847, 1993.

39. Overbeek, P.A., Lai S.P., VanQuill, K.R., and Westphal, H. Tissue-specific expression in transgenic mice of a fused gene containing RSV terminal sequences. Science 231:1574-1577, 1986.

40. Sympson, C.J., Talhouk, R.S., Alexander, C.M., Chin, J.R., Clift, S.M., Bissell, M.J., and Werb, Z. Targeted expression of stromelysin-1 in mammary gland provides evidence for a role of proteinases in branching morphogenesis and the requirement for an intact basement membrane for tissue-specific gene expression. J. Cell Biol. 125:681-693, 1994.

41. Gallahan, D., Jhappan, C., Robinson, G., Henninghausen, L., Sharp, R., Kordon, E., Callahan, R., Merlino, G., and Smith, G.H. Expression of a truncated int3 gene in developing secretory mammary epithelium specifically retards lobular differentiation resulting in tumorigenesis. Cancer Res. 56:1775-1785, 1996.

42. Lefebvre, O., Wolf, C., Limacher, J.M., Hutin, P., Wendling, C., LeMeur, M., Basset, P., and Rio, M.C. The breast cancer-associated stromelysin-3 gene is expressed during mouse mammary gland apoptosis. J. Cell Biol. 119:997-1002, 1992.

42a. Li, F., Strange, R., Friis, R.R., Djonov, V., Altermatt, H.J., Saurer, S., Niemann, H., and Andres, A.C. Expression of stromelysin-1 and TIMP-1 in the involuting mammary gland and in early invasive tumors of the mouse. Int. J. Cancer 59:560-568, 1994.

43. Neuenschwander, S., Schwartz, A., Wood, T.L., Roberts, C.T. Jr., Henninghausen, L., and LeRoith, D. Involution of the lactating mammary gland is inhibited by the IGF system in a transgenic mouse model. J. Clin. Invest. 97:2225-2232, 1996.

44. Pei, D., and Weiss, S.J. Transmembrane-deletion mutants of the membrane-type matrix metalloproteinase-1 activate progelatinase A and express intrinsic matrix-degrading activity. J. Biol. Chem. 271:9135-9140, 1996.

45. Seidah, N.G., and Chretien, M. Pro-protein convertases of subtilisin/kexin family. Meth. Enzymol. 244:175-188, 1994.

V. APPENDIX

1. Figure Legends and Figures

Figure 1. *Basal lamina synthesis by MDCK cells.* MDCK were cultured atop a layer of type I collagen in a Transwell culture system. Following a 21 day culture period in 10% FCS, the monolayer was fixed (panel A, x 5200) or lysed (panel B, x 7400) and processed for TEM. In panels C and D, the type I collagen gel was processed for SEM after a 21 day incubation in 10% FCS or after lysing a MDCK monolayer that had been cultured atop the collagen for 21 days, respectively (x 2000).

Figure 2. *Expression of ST-3 in stably transfected MDCK cell lines.* Stably transfected MDCK cell clones were expanded and the confluent monolayers switched to serum-free medium for 24 h. The conditioned media from MDCK cells transfected with the control vector (lane 1) or with ST-3 (lanes 2-7) were analyzed by immunoblotting with ST-3-specific polyclonal antisera. While two of the cell lines secreted small quantities of the zymogen (lanes 5 and 6), the bulk of the secreted enzyme was processed tots active form (labeled mature ST-3) or its derivative fragments (labeled ST-3 fragments).

Figure 3. *Basement membrane synthesis and invasion by ST-3 transfectants.* MDCK cells were stably transfected with human ST-3 and cultured atop a type I collagen matrix in a Transwell culture system for 3 weeks. Following this culture period, an intact basement membrane was observed (panels A and B, 8,300x and 15,600x, respectively). When cells were stimulated with recombinant scatter factor (50 ng/ml), the cells began to invade across the basement membrane (panel C, 25,000x) at day 5 and to form patent tubules (underneath the monolayer) in the underlying type I collagen gel by day 15 (panel D, 2,100x). The pattern of invasion was indistinguishable from that observed in control transfectants (data not shown).

Figure 4. *Southern blot of tail DNA from RSV LTR-ST-3 transgenic mice.* The structure of the transgene contains the RSV LTR promoter, the 1.5 kb ST-3 cDNA and the SV40 poly (A) signal. The size of the transgene is 2.7 kb. A unique KpnI site is located between RSV LTR and the ST-3 cDNA. Tail DNA (10 µg each) from control (lane 1) and ST-3 transgenic mice (lanes 2,3,4,5) were digested with KpnI, fractionated on 1% agarose gel, blotted, and the membrane hybridized to [^{32}P]-labeled human ST-3 probe. The 4 transgenic founders were identified from a total of 72 candidate mice. Copy numbers estimation: founder 1 (lane 2), 3 copies; founder 2 (lane 3), 4 copies; founder 3 (lane 4), 10 copies; founder 4 (lane 5), >20 copies.

Figure 5. *Southern blot of tail DNA from WAP-ST-3 transgenic mice.* Tail DNA (10 µg) from 8 founders (lanes 1-8) and 1 control (lane 9) were digested with Sac I, fractionated on a 1% agarose gel, blotted and the membrane hybridized to [^{32}P]-labeled human ST-3 probe. The 8 transgenic founders were identified from a total of 12 candidate mice.

Figure 6. *Preliminary analyses of ST-3 and WAP mRNA expression in transgenic mouse mammary gland.* Whole mammary gland RNA (10 µg) from virgin, late pregnant and lactating normal mice, and from late pregnant and lactating transgenic mice was separated on a 1% agarose gel in the presence of formaldehyde, transferred to a nylon membrane and hybridized to [^{32}P]-labeled probe for either human ST-3 or mouse WAP. After film exposure, the blots were stripped and rehybridized with the second labeled probe (either ST-3 or WAP). Normal mouse mammary glands at the stages presented do not express ST-3 (mouse ST-3 mRNA will hybridize with the human probe) which is first expressed starting around day 3 of involution (data not shown). Transgenic mice express ST-3 during late pregnancy and lactation coincident with WAP expression.

Figure 7. *Representative inguinal (#4) mammary glands removed from a normal virgin mouse (left column), a normal lactating mouse (center column) and a WAP-ST-3 transgenic mouse that was unable to maintain a litter of pups (right column).* The top and middle rows show respectively low and high magnification views of carmine stained whole-mount preparations, while the bottom row shows representative hematoxylin/eosin stained longitudinal sections. In comparison to the normal lactating gland, the transgenic gland is smaller overall. In addition, transgenic lobuloalveoli appear prematurely regressed, are much less densely packed than normal and often lack lumens. However, some large alveoli of the transgenic gland appear engorged with milk products.
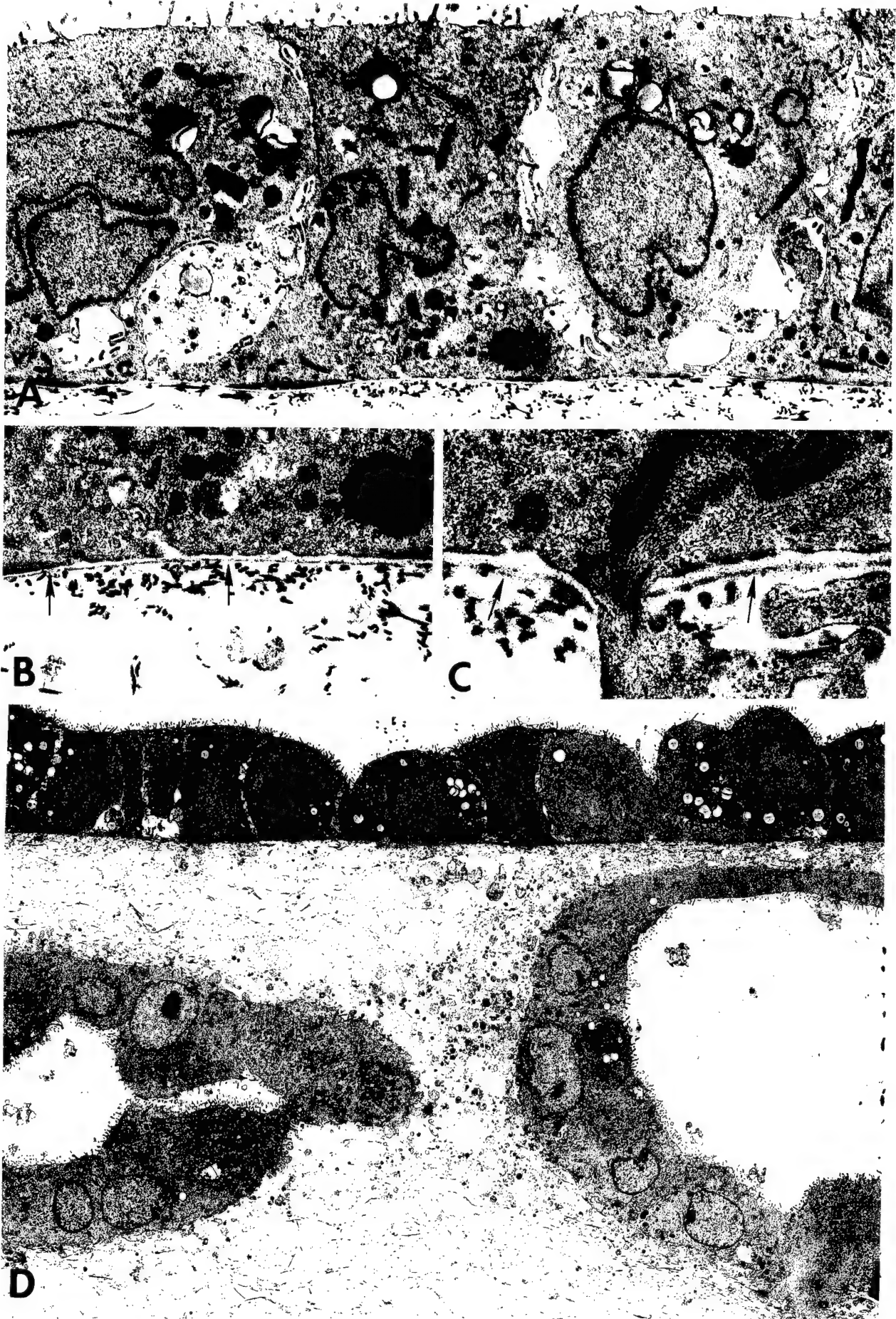
Figure 1
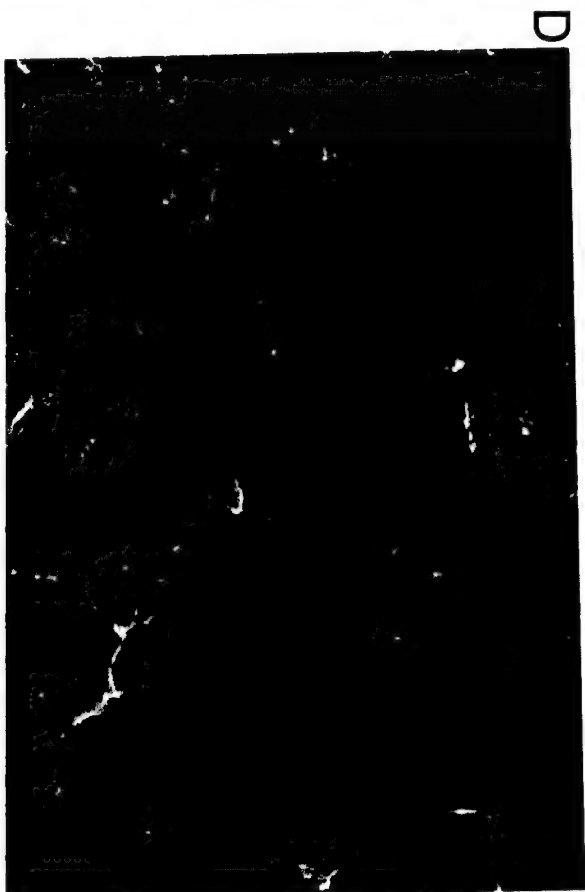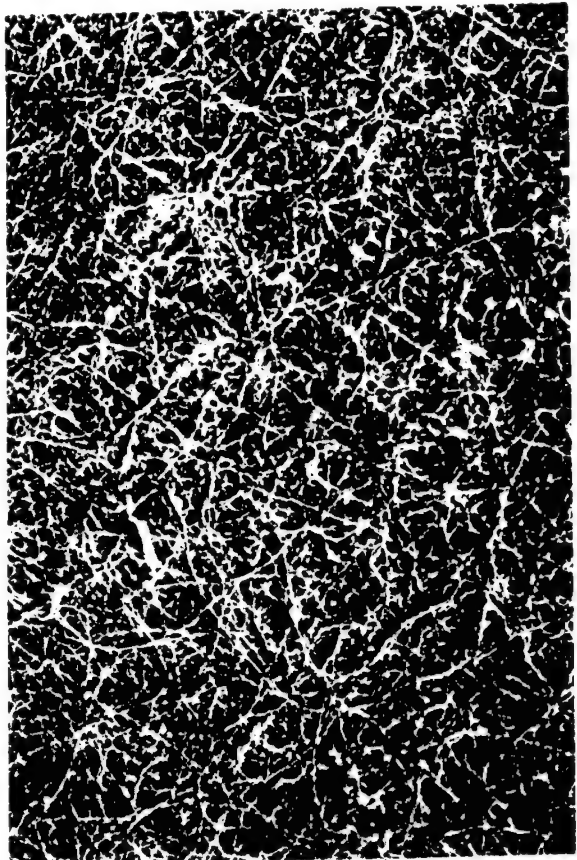
Figure 2

Expression of ST3
in MDCK Cells

←Pro-ST3

←Mature ST3

←ST3 fragments

1 2 3 4 5 6 7

Figure 3

Figure 4

Figure 5

Southern Blot of Tail DNA from
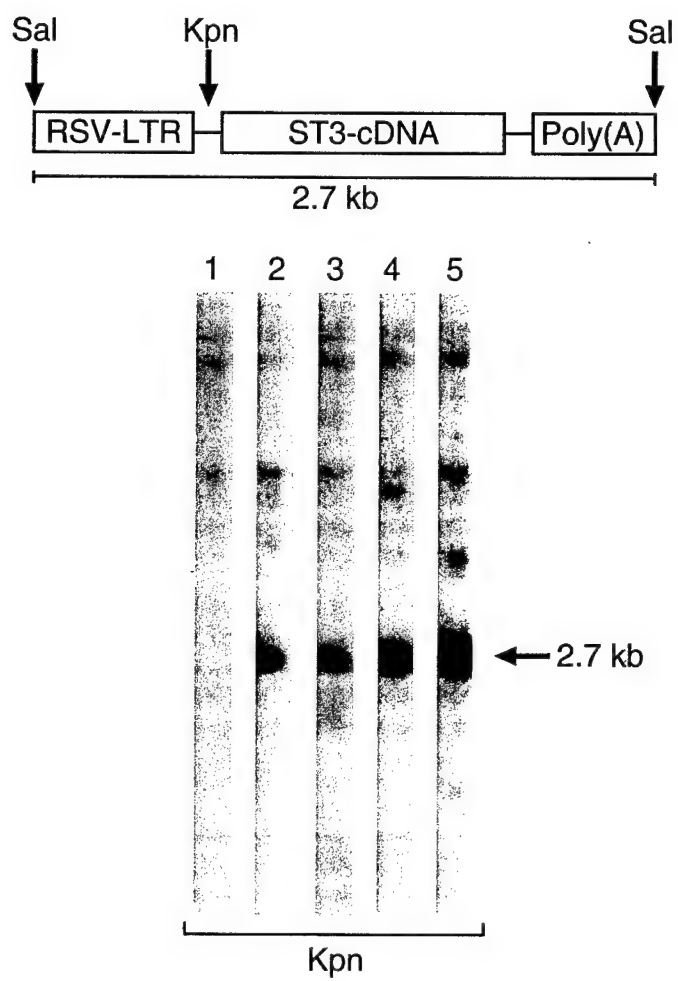WAP-ST3 Transgenic Mice
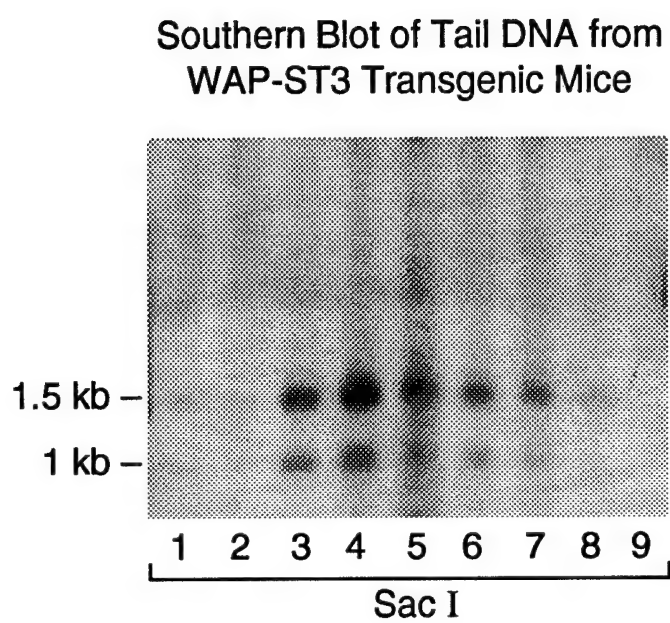

1.5 kb –

1 kb –

1 2 3 4 5 6 7 8 9

Sac I

Figure 6

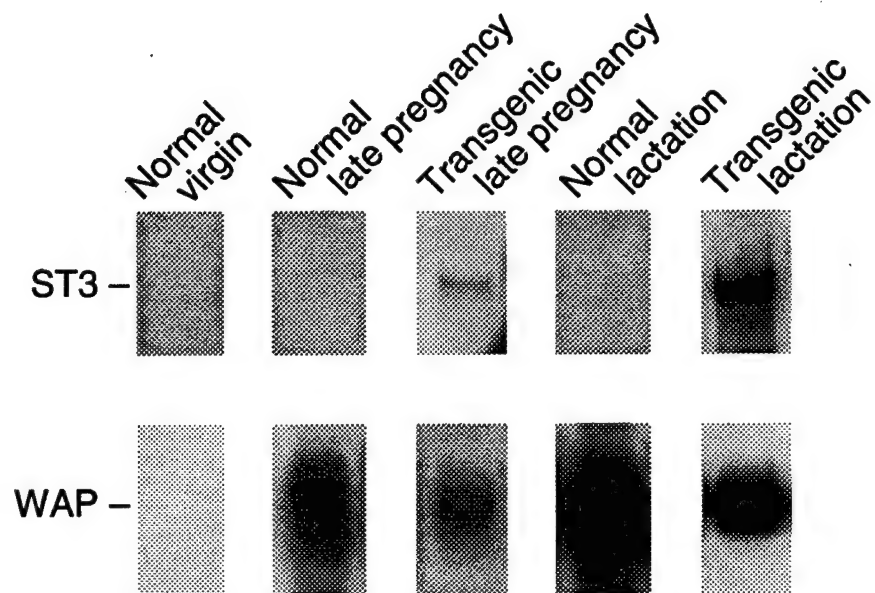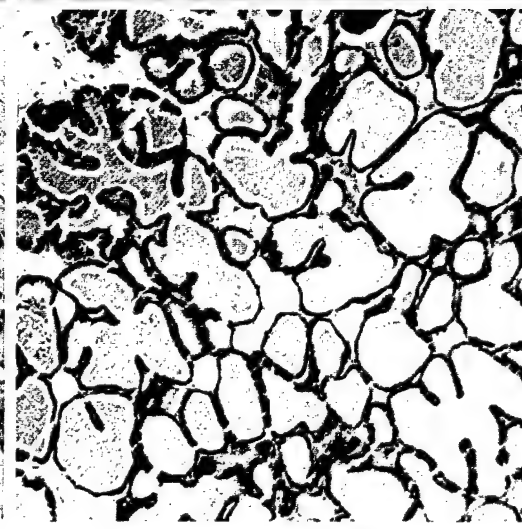# Preliminary Analyses of ST3 and
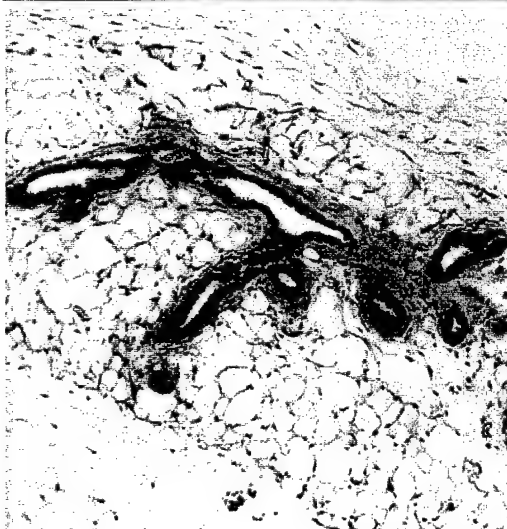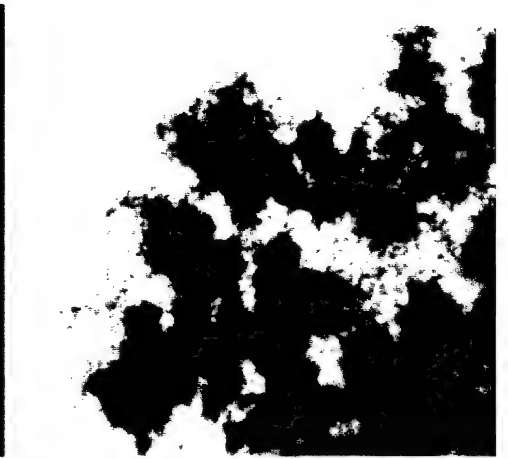# WAP mRNA Expression in Transgenics

Figure 7

Virgin
Control

Lactating
Control

"Lactating"
Transgenic

2. Publications

   a.   Pei, D., and Weiss, S.J. Transmembrane-deletion mutants of the membrane-type matrix metalloproteinase-1 activate progelatinase A and express intrinsic matrix-degrading activity. *J. Biol. Chem.* 1996, 271:9135-9140.

3. List of All Personnel Receiving Pay

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   c.   Susan Stevens, B.S.

Transmembrane-deletion Mutants of the Membrane-type Matrix Metalloproteinase-1 Process Progelatinase A and Express Intrinsic Matrix-degrading Activity*

(Received for publication, January 25, 1996)

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Membrane-type matrix metalloproteinase-1 (MT-MMP-1) has been proposed to play a critical role in regulating the expression of tissue-invasive phenotypes in normal and neoplastic cells by directly or indirectly mediating the activation of progelatinase A. To begin characterizing MT-MMP-1 structure-function relationships, transmembrane-deletion mutants were constructed, and the processing of the zymogens as well as the enzymic activity of the mature proteinases was analyzed. We now demonstrate that pro-MT-MMP-1 mutants are efficiently processed to active proteinases following post-translational endoproteolysis immediately downstream of an Arg^{108}-Arg-Lys-Arg basic motif by a proprotein convertase-dependent pathway. The secreted form of active MT-MMP-1 not only displays an N terminus identical with that described for the processed wild-type enzyme at Tyr^{112} (Strongin, A. Y., Collier, I., Bannikov, G., Marmer, B. L., Grants, G. A., and Goldberg, G. I. (1995) *J. Biol. Chem.* 270, 5331–5338), but also directly mediated progelatinase A activation via a two-step proteolytic cascade indistinguishable from that observed with intact cells. Furthermore, although the only function previously ascribed to MT-MMP-1 is its ability to act as a progelatinase A activator, purified transmembrane deletion mutants also expressed proteolytic activities against a wide range of extracellular matrix molecules. Given recent reports that MT-MMP-1 ectodomains may undergo intercellular transfer *in vivo* (Okada, A., Bellocq, J.-P., Rouyer, N., Chenard, M.-P., Rio, M.-C., Chambon, P., and Basset, P. (1995) *Proc. Natl. Acad. Sci. U. S. A.* 92, 2730–2734), our data suggest that soluble forms of the proteinase confer recipient cells with the ability to not only process progelatinase A, but also directly degrade extracellular matrix components.

Members of the matrix metalloproteinase (MMP)[1] gene family have been implicated in the physiologic as well as pathologic

remodeling of the extracellular matrix (ECM) in events ranging from organogenesis to tumor metastasis (1–3). Very recently, three new members of this family were discovered by screening cDNA libraries for homologies to conserved regions of the known MMP genes and named the membrane-type matrix metalloproteinases-1, -2, and -3 (MT-MMP-1, -2, and -3; Refs. 4–6). Based on their predicted amino acid sequences, each of the MT-MMPs, like almost all previously characterized MMPs, contains (i) a candidate leader sequence, (ii) a propeptide region which includes a highly conserved PRCGXPD sequence that helps stabilize the MMP zymogen in a catalytically inactive state, (iii) a zinc-binding catalytic domain, and (iv) a hemopexin-like domain near their respective C termini (4–7). In addition, in a pattern similar to that described for stromelysin-3, each of the MT-MMPs contains a short amino acid insert sandwiched between their pro- and catalytic domains that encodes a potential recognition motif for members of the proprotein convertase family (4–8). Despite their considerable similarity to other MMP family members,[2] however, only the MT-MMPs contain ~75–100 amino acid extensions at their C termini, each of which includes a hydrophobic stretch consistent with the presence of a transmembrane (TM) domain (4–6, 9). Thus, in contradistinction to all other MMPs, the MT-MMPs are expressed as membrane-associated ectoenzymes rather than soluble proteins.

Although little is known with regard to the potential functions of the MT-MMPs, most attention has focused on the ability of MT-MMP-1 as well as MT-MMP-3 to induce the processing of the MMP zymogen, progelatinase A, to its activated form (*i.e.* $[Tyr^{81}]$gelatinase A) via a $[Leu^{38}]$gelatinase A intermediate (4, 6, 10). Given the ability of activated gelatinase A to cleave a wide range of ECM substrates (including native types I, IV, V, VII, and XI collagen, denatured collagens, elastin, proteoglycans, laminin, and fibronectin) as well as the association of gelatinase A activation with the expression of tissue-invasive phenotypes (1–3, 11–15), MT-MMPs have been dubbed as possible "master switches" that control ECM remodeling (16). Nonetheless, the processes that regulate the activation of the MT-MMP zymogens themselves to mature forms remain undefined as does the mechanism by which MT-MMPs mediate progelatinase A activation (4–6, 9, 10). In large part, further progress in characterizing MT-MMP activities has been hindered by the technical problems associated with isolating and purifying membrane-associated molecules. Given that similar difficulties with other transmembrane enzymes have been negotiated by generating TM-deleted soluble mutants (17–19), we noted that, with the exception of the extended C-terminal domain, the modular organization of the MT-MMPs is identical with that of the secreted MMPs (7). Hence, two

[1] The abbreviations used are: MMP, matrix metalloproteinase; α_1PI, α_1-proteinase inhibitor; α_1PI$_{Pitt}$, Pittsburgh mutant of α_1-proteinase inhibitor; ECM, extracellular matrix; MT-MMP-1, -2, -3, membrane-type matrix metalloproteinase-1, -2, and -3; ΔMT-MMP-1, transmembrane-deleted MT-MMP-1; MT-MMP-1$_{ΔA}$, MT-MMP-1$_{1-538}$; MT-MMP-

1$_{ΔB}$, MT-MMP-1$_{1-508}$; TM, transmembrane; MDCK, Madin-Darby canine kidney cells; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction.

[2] All MT-MMPs also contain a homologous 8-amino acid insert within their catalytic domains whose function remains undefined (4–6).

TM-deletion mutants of MT-MMP-1 were constructed by either truncating the molecule (i) immediately upstream of the start site of the TM domain (*i.e.* MT-MMP-1$_{1-538}$; herein referred to as MT-MMP-1$_{\Delta A}$) or (ii) at the conserved cysteinyl residue found at, or near, the terminus of all hemopexin domain-containing MMPs (*i.e.* MT-MMP-1$_{1-508}$ or MT-MMP-1$_{\Delta B}$). Utilizing these constructs, we now demonstrate that TM-deleted MT-MMP-1 (ΔMT-MMP-1) mutants undergo efficient post-translational endoproteolysis between Arg^{111}-Tyr^{112} by a proprotein convertase-dependent pathway to generate fully active proteinases. Furthermore, the purified ΔMT-MMP-1 mutants not only activate recombinant progelatinase A directly via a two-step activation cascade identical with that described for the membrane-associated enzyme, but they also express heretofore unsuspected ECM-degrading activities.

EXPERIMENTAL PROCEDURES

Cell Culture—COS-7 cells and MDCK cells (both obtained from ATCC) were maintained in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) containing 10% fetal bovine serum (HyClone) and 4 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin as described (8, 20). Cells were transfected with purified plasmid DNA by LipofectAMINE treatment (Life Technologies Inc.). MDCK clones stably expressing MT-MMP-1$_{\Delta B}$ were established by transfection and subsequently selected with G418 (Life Technologies, Inc.) as described previously (8, 20).

Plasmids—Following screening of an HT-1080 cDNA library constructed in the pBK-CMV vector with a 0.45-kilobase canine MT-MMP-1 cDNA isolated from an MDCK cDNA library,[3] a human clone was obtained and sequenced. The predicted amino acid sequence of full-length HT-1080 MT-MMP-1 was identical with that described by Okada *et al.* (13). To generate pro-MT-MMP-1$_{\Delta A}$ and pro-MT-MMP-1$_{\Delta B}$, a 5′ primer (ACCATGTCTCCCGCCCCAAGACCCTCCCGT) was paired with the 3′ primers, TTCAGCTCACCGCCCCGCCGCC or TTCAGAA-GAAGAAGACTGCAAGGCC, respectively, in separate PCR reactions to generate cDNA fragments with the intended truncations at the C terminus (*i.e.* immediately prior to the TM domain as defined by Cao *et al.* (9) at Ser^{538} or at the conserved Cys residue at position 508 (6, 7); see Fig. 1A). The PCR fragments were then cloned into pCR3-Uni vector (Invitrogen) and characterized by sequencing as described (8). Expression vectors for α$_1$-proteinase inhibitor (α$_1$PI), the Pittsburgh mutant of α$_1$PI (α$_1$PI$_{Pitt}$), and a TM-deletion mutant of furin were provided by A. Rehemtulla and R. Kaufman (University of Michigan).

Mutagenesis—Sequential PCR-based mutagenesis was performed as described previously, and the mutagenized fragments were cloned into pCR3-Uni vector and characterized by sequencing (8). The mutagenic primers used are as follows: ATCAAGGCCAATGTT**GCA**AGGAAGC-GCTACGCC for R108A, GCCAATGTTCGAAGG**GCG**CGCTACGCCA-TCCAG for K110A, AATGTTCGAAGGAAG**GCC**TACGCCATCCAG-GGT for R111A, and GTTCGAAGGAAGC GC**TTT**GCCATCCAGGGG-TCTC for Y112F (bold nucleotides indicate the altered codons). MT-MMP-1$_{\Delta B}$ expression constructs as well as those harboring the above mutations (1 μg each) were transiently transfected into COS-7 cells (2 × 10⁵/ml) by LipofectAMINE treatment, and, after a 24-h incubation, an aliquot of the cell-free supernatant (0.02 ml) was analyzed by Western blotting as described below.

Protein Purification—MDCK cells stably expressing MT-MMP-1$_{\Delta B}$ were incubated in Opti-MEM (Life Technologies Inc.) supplemented with the synthetic MMP inhibitor, BB-94 (0.5 μM, British Biotechnology; Ref. 21), to trap the active form of the proteinase as a reversible enzyme-inhibitor complex (20). After 48 h, ~2 liters of conditioned media were collected, dialyzed against buffer A (50 mM Tris, pH 7.5, 5 mM CaCl₂), and then loaded onto a Q-Sepharose column (1.5 × 10 cm). Bound material was eluted with a NaCl gradient (0 to 1 M), and fractions containing MT-MMP-1$_{\Delta B}$ (identified by Western blotting) were combined and dialyzed against buffer A. A heparin-Sepharose column (1 × 10 cm) was then loaded with the dialyzed materials and developed with a NaCl gradient (0 to 1 M). Positive fractions were pooled and passed through a gelatin-Sepharose column (1 × 5 cm) followed by gel filtration chromatography (Ultrogel ACA44, 1 × 150 cm) in the absence of BB-94 to regenerate the active proteinase. In selected experiments, MT-MMP-1$_{\Delta A}$ was purified from a batch culture of transiently transfected MDCK cells as described above.

[3] D. Pei and S. J. Weiss, unpublished observation.
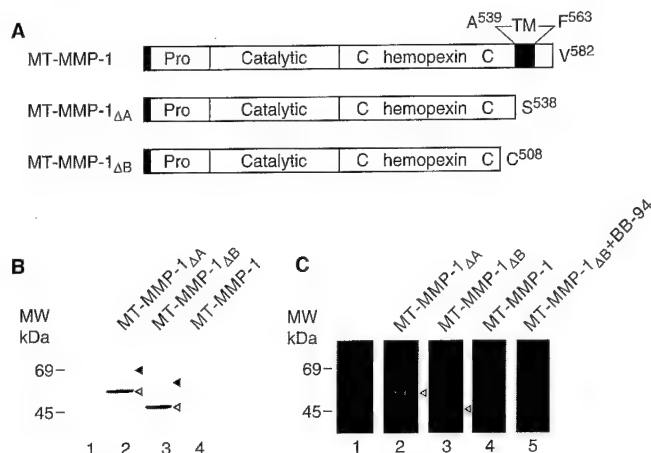
FIG. 1. **Expression and characterization of ΔMT-MMP-1.** *A*, domain alignments of MT-MMP-1 and the ΔMT-MMP-1 mutants. Wild-type MT-MMP-1 contains 582 amino acids arranged as a series of pre- (*shaded box*), pro-, catalytic, hemopexin (bounded by a pair of highly conserved cysteinyl residues indicated as *C*), TM (*shaded in black* and marked *TM*), and cytosolic (residues 564 to 582) domains. MT-MMP-1$_{\Delta A}$ is truncated at the edge of the TM domain while MT-MMP-1$_{\Delta B}$ ends at the conserved cysteinyl residue that marks the extreme C terminus of the hemopexin domain. *B*, Western blot analysis of ΔMT-MMP-1-transfected cells. Serum-free conditioned medium from COS-7 cells transiently transfected with control (*lane 1*), MT-MMP-1$_{\Delta A}$ (*lane 2*), MT-MMP-1$_{\Delta B}$ (*lane 3*), or wild-type MT-MMP-1 (*lane 4*) expression vectors were analyzed by immunoblotting with MT-MMP-1-specific polyclonal antisera. The *dark* and *clear arrowheads* indicate the positions of the putative pro- and processed forms of ΔMT-MMP-1. In wild-type MT-MMP-1-transfected cells, immunoblots of cell lysates identified the 63-kDa form of the enzyme as the major product (data not shown). *C*, gelatin zymography of MT-MMP-1-transfected cells. Serum-free conditioned medium from COS-7 cells transiently transfected with control (*lane 1*), MT-MMP-1$_{\Delta A}$ (*lane 2*), MT-MMP-1$_{\Delta B}$ (*lane 3*), or MT-MMP-1 (*lane 4*) expression vectors were depleted of endogenous progelatinase A by gelatin affinity chromatography and analyzed by zymography. In *lane 5*, the MT-MMP-1$_{\Delta B}$ zymogram was developed in the presence of 1.0 μM BB-94. Identical results were obtained with β-casein or κ-elastin zymograms (D. Pei and S. J. Weiss, unpublished observation).

SDS-PAGE, Western Blots, Zymography, and N-terminal Sequencing—Basic protocols for these techniques have been described (8, 20). The MT-MMP antisera were raised in New Zealand rabbit using a bacterially generated recombinant fusion protein between glutathione transferase and MT-MMP$_{91-243}$ (20).

Enzymic Reactions—Enzyme assays of MT-MMP-1$_{\Delta A}$ and MT-MMP-1$_{\Delta B}$ were performed in buffer A supplemented with 150 mM NaCl at 37 °C unless noted otherwise. Matrix substrates (devoid of contaminating progelatinase A activity as determined by gelatin zymography; data not shown) were obtained from Collaborative Research (type I, IV, and V collagens, fibronectin, laminin, vitronectin, and dermatan sulfate proteoglycan). Processing of pro-MT-MMP-1$_{\Delta B}$ by purified soluble furin (specific activity 500 units/μg; Ref. 18) was performed as described (8). Recombinant progelatinase A (purified as described in Ref. 22) was a gift from R. Fridman (Wayne State University, Detroit, MI), recombinant human TIMP-2 was supplied by Amgen, and soluble human furin was provided by R. Fuller, University of Michigan.

RESULTS

Expression of ΔMT-MMP-1 Mutants and Detection of Enzymic Activity—COS-7 cells transiently transfected with either MT-MMP-1$_{\Delta A}$ or MT-MMP-1$_{\Delta B}$ cDNA each secreted a pair of major and minor products that were specifically recognized by polyclonal antibodies to a truncated form of the bacterially expressed protein (Fig. 1B). While the molecular mass of the minor secreted proteins (~64 kDa for MT-MMP-1$_{\Delta A}$ and ~60 kDa for MT-MMP-1$_{\Delta B}$; lanes 2 and 3, respectively) were consistent with those of the predicted proforms of the metalloproteinases, the major soluble species detected with either TM-deletion mutant was a fragment ~10 kDa smaller in size. The generation of the major and minor forms was not specific to COS-7 cells since a similar profile was generated with trans-
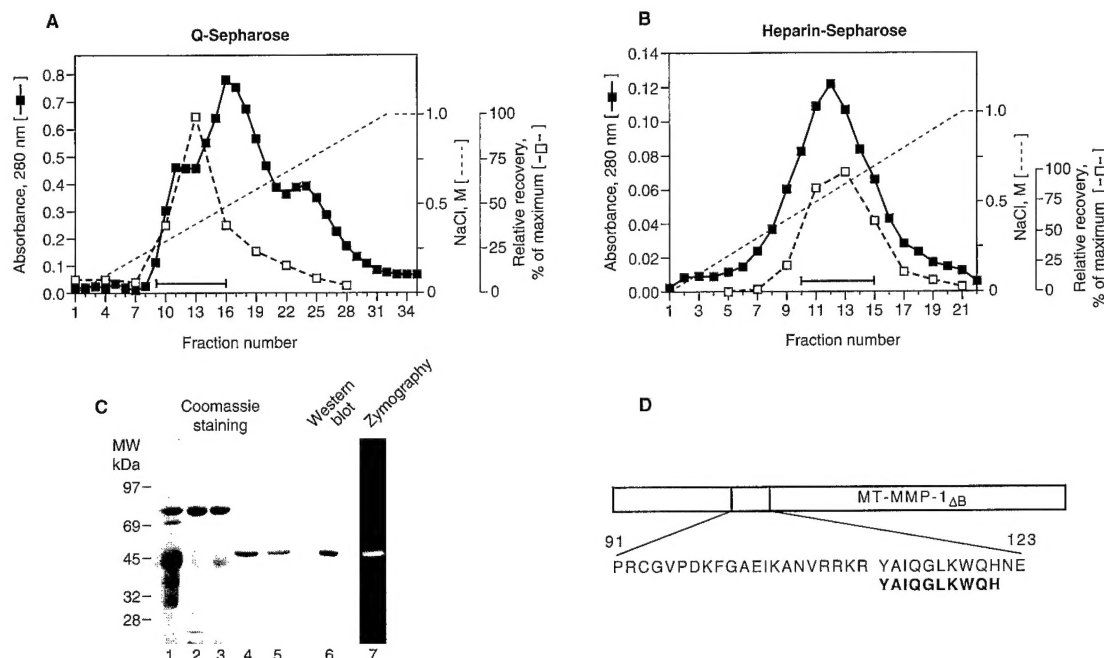
FIG. 2. **Purification of MT-MMP-1$_{\Delta B}$.** *A* and *B*, fractionation of MT-MMP-1$_{\Delta B}$ on Q-Sepharose and heparin-Sepharose, respectively. Conditioned media from MDCK cells stably transfected with MT-MMP-1$_{\Delta B}$ were loaded onto a Q-Sepharose column and eluted with a NaCl gradient. The protein content of each fraction was monitored at A_{280} (*dark squares*) while MT-MMP-1$_{\Delta B}$ content in each fraction was monitored by immunoblot analysis and reported as the percent recovered relative to the fraction containing the highest concentration of MT-MMP-1$_{\Delta B}$ (*open squares*). Fractions 9–16 were combined, dialyzed against buffer A, loaded onto a heparin-Sepharose column, and eluted with a NaCl gradient. *C*, characterization of the isolated MT-MMP-1$_{\Delta B}$ products. Conditioned media (*lane 1*), a pool of fractions 9–16 eluted from Q-Sepharose (*lane 2*), flow-through of fractions 9–16 that did not bind to heparin-Sepharose (*lane 3*), pool of fractions 10–15 eluted from heparin-Sepharose column (*lane 4*), and final purified form of MT-MMP-1$_{\Delta B}$ (4.5 pmol; *lane 5*) were separated by SDS-PAGE and visualized by Coomassie staining. In *lanes 6* and *7*, purified MT-MMP-1$_{\Delta B}$ (1.2 pmol) was analyzed by immunoblotting and gelatin zymography, respectively. *D*, the N terminus of MT-MMP-1$_{\Delta B}$ as determined after 10 cycles of sequencing (indicated by *bold letters*). The *open box* represents the MT-MMP-1$_{\Delta B}$ open reading frame with the amino acid sequence of MT-MMP-1 from Pro^{91} to Glu^{123}.
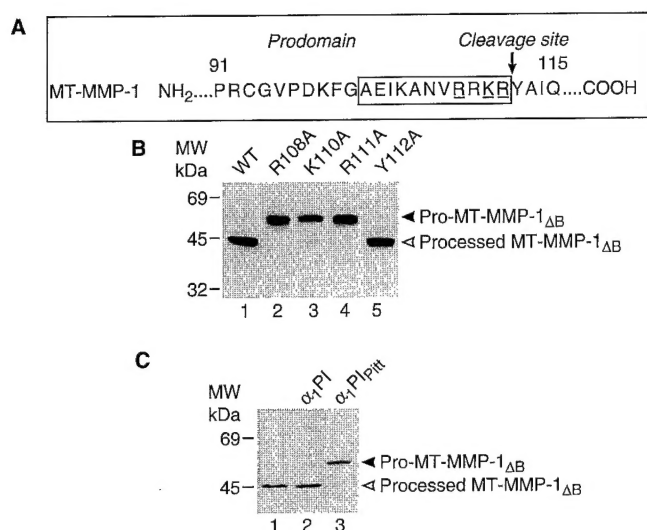
fected MDCK cells.[3] As expected, when cells were transiently transfected with wild-type MT-MMP-1, soluble forms of the enzyme were not detected in the conditioned media (Fig. 1*B*).

In intact cell systems, MMPs can be recovered in conditioned medium as a mixture of zymogens, processed active enzymes, zymogen-inhibitor complexes, or enzyme-inhibitor complexes (1–3). In the case of almost all MMP family members, many of these forms can be detected following SDS-PAGE in substrate-impregnated gels (1–3). Thus, serum-free conditioned media from control, MT-MMP-1$_{\Delta A}$, MT-MMP-1$_{\Delta B}$, or MT-MMP-1 transfected cells were depleted of endogenous gelatinases by gelatin affinity chromatography (ΔMT-MMP-1 does not bind to gelatin; see below) and electrophoresed in gelatin- containing gels. Proteinases were then allowed to renature following the removal of SDS and then incubated overnight at 37 °C. As shown in Fig. 1*C*, supernatants recovered from MT-MMP-1$_{\Delta A}$ or MT-MMP-1$_{\Delta B}$-transfected cells each revealed the presence of a single band of gelatinolytic activity whose relative mobility matched that of the major form of ΔMT-MMP-1 detected by Western blotting. Significantly, identical results were obtained when zymograms were performed with either β-casein- or κ-elastin-impregnated gels as well (data not shown). Regardless of substrate used, the band of proteolytic activity attributed to either ΔMT-MMP-1 mutant was completely inhibited when zymograms were performed in the presence of the MMP-specific inhibitor, BB-94 (Fig. 1*C*).

Purification of ΔMT-MMP-1 and Characterization of Zymogen Processing—Because both MT-MMP-1$_{\Delta A}$ and MT-MMP-1$_{\Delta B}$ appeared to undergo a similar, if not identical, processing event to generate active proteinases as assessed by zymography, one of the mutants (*i.e.* MT-MMP-1$_{\Delta B}$) was stably expressed in the MDCK cell line for further analyses. As shown in Fig. 2, serum-free conditioned media from stable transfectants expressing MT-

MMP-1$_{\Delta B}$ were subjected to a combination of gelatin-Sepharose affinity, Q-Sepharose affinity, and heparin-Sepharose affinity chromatography followed by gel filtration chromatography. Utilizing this protocol, a single immunoreactive ~50-kDa species was isolated that co-migrated with the band of activity detected by gelatin-zymography (Fig. 2*C*). Following 10 cycles of N-terminal sequence analysis, the material was identified as MT-MMP-1 with a single start site at Tyr^{112} (Fig. 2*D*). Interestingly, this N terminus not only aligns with that of the active forms of all other MMPs (6), but it is also identical with that reported for the mature form of wild-type MT-MMP-1 recovered from the HT-1080 fibrosarcoma cell line (10). Thus, while almost all MMPs are synthesized and secreted as inactive zymogens, MT-MMP-1$_{\Delta B}$ (as well as MT-MMP-1$_{\Delta A}$; see below) underwent further processing to its active form.
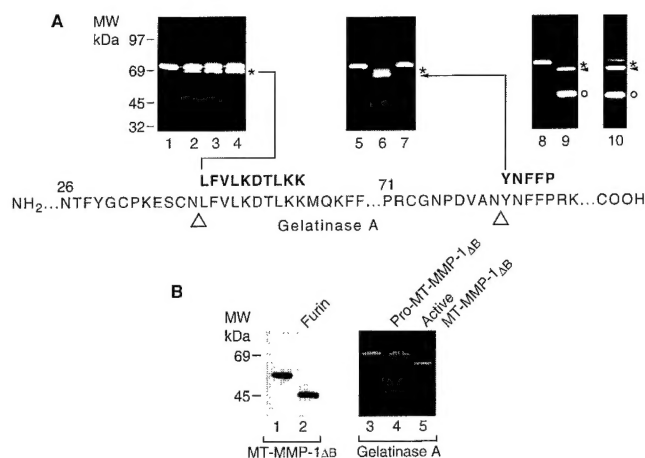
Like stromelysin-3, the only other membrane of the MMP family to be secreted as a fully processed active proteinase, MT-MMP-1 contains a motif of basic amino acids (*i.e.* ^{114}RRKR) immediately upstream of its catalytic domain (see Fig. 3*A*; Refs. 4 and 8). Recently, the R*X*KR array in stromelysin-3 (*X* = nonbasic amino acid) was shown to act as an endoproteolytic processing signal for an intracellular serine proteinase belonging to the proprotein convertase family (8). Because specific proprotein convertases can display varying requirements for basic residues at positions -1, -2, and -4 relative to the scissile bond (*i.e.* P^{-1}, P^{-2}, and P^{-4}, respectively; Refs. 23 and 24), a potential role for this enzyme class in ΔMT-MMP-1 processing was initially assessed by successively substituting each basic residue with an Ala moiety in transient transfection assays. As shown in Fig. 3*B*, each of these substitutions almost completely blocked MT-MMP-1$_{\Delta B}$ processing (*lanes 2–4*). In contrast, a Tyr $\rightarrow$ Ala substitution at the less critical P^{+1} site (23) did not affect MT-MMP-1$_{\Delta B}$ processing (Fig. 3, *lane 5*).

A

Prodomain   *Cleavage site*
        91   ↓ 115
MT-MMP-1 NH₂....PRCGVPDKFG[AEIKANVRRKR]YAIQ....COOH

B

C

FIG. 3. **Proprotein convertase-dependent processing of MT-MMP-1ₐB.** *A*, mutational analysis of the putative proprotein convertase recognition motif. The amino acids surrounding the cleavage site in MT-MMP-1ₐB are shown with the basic residue motif *underlined. B*, expression vectors for native MT-MMP-1ₐB (*lane 1*), Arg¹⁰⁸ → Ala (*R108A; lane 2*), Lys¹¹⁰ → Ala (*K110A; lane 3*), Arg¹¹¹ → Ala (*R111A; lane 4*), and Tyr¹¹² → Ala (*Y112A; lane 5*) were transfected into COS-7 cells (2 × 10⁵/ml) by LipofectAMINE treatment, and the secreted products (0.02 ml of the cell-free supernatant) were analyzed by immunoblotting. *C*, inhibition of MT-MMP-1ₐB processing by α₁PIₚᵢₜₜ. COS-7 cells were transiently transfected as described above with MT-MMP-1ₐB expression vector alone (*lane 1*) or co-transfected with MT-MMP-1ₐB and α₁PI (*lane 2*) or α₁PIₚᵢₜₜ expression vectors (*lane 3*), and the cell-free supernatants were analyzed by immunoblotting.

Given that COS are known to express only two members of the proprotein convertase family that recognize RXKR motifs (*i.e.* furin and PACE4; Ref. 24), cells were co-transfected with MT-MMP-1ₐB and the Pittsburgh mutant of α₁PI (α₁PIₚᵢₜₜ), a reactive site variant that inhibits furin (but not PACE4) activity *in situ* (25, 26). Under these conditions, α₁PIₚᵢₜₜ completely blocked MT-MMP-1ₐB processing while wild-type α₁PI exerted no inhibitory effect (Fig. 3*C*). MT-MMP-1ₐB processing was similarly inhibited by α₁PIₚᵢₜₜ in MDCK cells (data not shown). These results (together with the demonstration that soluble furin processes pro-ΔMT-MMP-1 to its active form under cell-free conditions; see below) indicate that pro-ΔMT-MMP-1 maturation is regulated by a furin-dependent pathway in an intact cell system.

Activation of Recombinant Progelatinase A by Purified ΔMT-MMP-1—Current evidence indicates that MT-MMP-1-expressing cells initiate progelatinase A activation via a two-step process that involves an initial cleavage of the Asn³⁷-Leu bond followed by an autocatalytic conversion of the Leu³⁸ intermediate into a 62-kDa active enzyme with an N-terminal Tyr⁸¹ residue (10, 27). Nonetheless, the ability of MT-MMP to directly cleave progelatinase A is unclear, and it has been postulated that additional intermediates may be involved (9, 10). Thus, purified active MT-MMP-1ₐB was incubated with recombinant progelatinase A and processing monitored by gelatin zymography and N-terminal sequence analysis. Following a 2-h incubation at 37 °C, MT-MMP-1ₐB initially cleaved progelatinase A (which migrates as a ~68-kDa species) into a ~64-kDa fragment (Fig. 4*A*). N-terminal sequence analysis of the ~64-kDa gelatinase A fragment yielded the Leu³⁸ form of the enzyme (Fig. 4*A*). Subsequently, the 64-kDa form of the enzyme underwent further processing to a 62-kDa product whose N terminus confirmed the generation of [Tyr⁸¹]gelatinase A (Fig. 4*A*). As expected, the ability of MT-MMP-1ₐB (as well as MT-MMP-1ₐₐ; data not shown) to activate progelatinase A was

A

26
NH₂...NTFYGCPKESCNLFVLKDTLKKMQKFF...PRCGNPDVANYNFFPRK...COOH
          Gelatinase A

B

FIG. 4. **ΔMT-MMP-1-dependent activation of recombinant progelatinase A.** *A*, zymography and N-terminal sequence analysis of progelatinase A. Recombinant progelatinase A (140 nM) was incubated alone (*lane 1*) or with 14 nM, 28 nM, or 56 nM MT-MMP-1ₐB (*lanes 2–4*, respectively) for 2 h at 37 °C in buffer A supplemented with 150 mM NaCl and 0.01% Brij 35 in a final volume of 0.02 ml. In *lanes 5–7*, progelatinase was incubated alone, with MT-MMP-1ₐB or with MT-MMP-1ₐB and TIMP-2 (350 nM), respectively, for 16 h at 37 °C. Aliquots of each reaction mixture were analyzed by gelatin zymography or were electrophoresed and electroblotted for N-terminal sequence analysis. The *bold sequence* beginning with Leu³⁸ represents the first 10 cycles of the N terminus of the ~64-kDa form of gelatinase A (indicated by *asterisk* in *lane 4*) while the sequence beginning with Tyr¹¹² represents the first 5 cycles of the N terminus of the ~62-kDa form of gelatinase (indicated by *dark arrow* in *lane 7*). The faint bands of gelatinolytic activity detected at ~50 kDa are due to MT-MMP-1ₐB (*lanes 2–4*). In *lanes 8–10*, progelatinase A (140 nM) was incubated alone (*lane 8*) with MT-MMP-1ₐB (14 nM; *lane 9*) or MT-MMP-1ₐₐ (14 nM; *lane 10*) for 3 h in 20 mM Hepes/KOH (pH 7.5), 0.1 mM CaCl₂, and 0.02% Brij-35 as described (10). The *asterisk, arrowhead,* and *circle* indicate the positions of the ~64-kDa, ~62-kDa, and ~42-kDa forms of gelatinase A, respectively. *B*, furin-mediated processing of pro-MT-MMP-1ₐB. Pro-MT-MMP-1ₐB (100 nM) isolated from α₁PIₚᵢₜₜ co-transfected cells was incubated alone (*lane 1*) or with soluble furin (10 nM; *lane 2*) for 1 h at 37 °C in a final volume of 0.02 ml and analyzed by Western blotting. For zymography (*lanes 3–5*), progelatinase A (140 nM) was incubated alone (*lane 3*), with pro-MT-MMP-1ₐB (28 nM; *lane 4*), or with furin-processed MT-MMP-1ₐB (28 nM; *lane 5*) for 16 h at 37 °C. Furin alone did not activate progelatinase A.

completely blocked by the addition of the MMP inhibitor, TIMP-2 (Fig. 4*A*) or BB-94 (data not shown). Interestingly, while earlier studies have demonstrated that membrane-associated forms of MT-MMP-1 can also process progelatinase A into a 42-kDa active species (10, 27, 28), this product was not detected under the standard conditions employed. However, when either MT-MMP-1ₐₐ or MT-MMP-1ₐB were incubated with progelatinase A in a low ionic strength buffer identical with that used previously (10, 27, 28), MT-MMP-1ₐₐ/B-dependent progelatinase A activation was significantly accelerated and the 42-kDa form of gelatinase generated (Fig. 4*A, lanes 8–10*).

The ability of mature MT-MMP-1ₐₐ/B to mediate progelatinase A activation is consistent with a model wherein active MT-MMP-1ₐₐ/B directly cleaves the gelatinase zymogen, but the data do not rule out the possibility that the ΔMT-MMP-1 zymogen only induces progelatinase A to undergo autocatalytic processing to its active form. Hence, purified pro-ΔMT-MMP-1 was isolated (*i.e.* from cells co-transfected with MT-MMP-1ₐB and α₁PIₚᵢₜₜ), and its ability to mediate progelatinase A activation was examined. As shown in Fig. 4*B*, pro-MT-MMP-1ₐB was unable to stimulate progelatinase A activation. However, when pro-MT-MMP-1ₐB was processed to its active form *ex situ* with a TM-deleted soluble form of furin (Fig. 4*B, lane 2*) and then incubated with progelatinase A, the gelatinase zymogen was readily activated (*lane 5*). Thus, only the processed active form
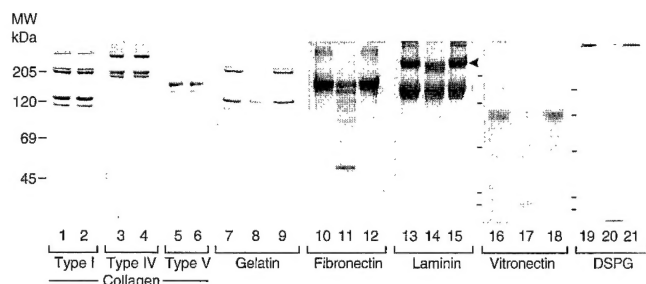
FIG. 5. **Substrate specificity of MT-MMP-1$_{\Delta B}$.** Type I collagen (3 μg; *lane 1*), type IV collagen (2 μg; *lane 3*), and type V collagen (2 μg; *lane 5*) were incubated alone or with 45 nM MT-MMP-1$_{\Delta B}$ (*lanes 2, 4, and 6*, respectively) at 25 °C for 16 h. Type I gelatin (3 μg; *lane 7*), fibronectin (4 μg; *lane 10*), laminin (4 μg; *lane 13*), vitronectin (4 μg; *lane 16*), or dermatan sulfate proteoglycan (5 μg; *lane 19*) were incubated alone, with MT-MMP-1$_{\Delta B}$ (45 nM; *lanes 8, 11, 14, 17,* and *20*, respectively) or with MT-MMP-1$_{\Delta B}$ and 1 μM BB-94 (*lanes 9, 12, 15, 18,* and *21*, respectively) at 37 °C in a final volume of 0.025 ml for 16 h. Reaction mixtures were separated by SDS-PAGE and Coomassie-stained. The *arrowhead* by *lane 15* indicates the position of the laminin B chain, while the *hatch marks* at the margin of *lanes 16* and *19* indicate the positions of the 97-, 69-, 45-, 32-, and 28-kDa molecular mass markers, respectively. Dermatan sulfate proteoglycan is recorded as *DSPG*.

of ΔMT-MMP-1 is able to mediate progelatinase A activation.

ECM-degrading Activity of ΔMT-MMP-1—While previous attention has focused solely on the role of MT-MMP-1 in progelatinase A activation (4, 9, 10, 13, 27), the ability of ΔMT-MMP-1 to degrade gelatin, β-casein, or κ-elastin following zymography suggested that the proteinase might express activity against a wider range of targets. Thus, purified MT-MMP-1$_{\Delta A/B}$ was incubated with either basement membrane- or interstitium-associated ECM molecules, and proteolysis was assessed in the absence or presence of BB-94. As shown in Fig. 5, while MT-MMP-1$_{\Delta B}$ did not degrade native type I, IV, or V collagens, the enzyme readily proteolyzed gelatin as well as fibronectin, the B chain of laminin, vitronectin, and dermatan sulfate proteoglycan via a BB-94-sensitive process. Similar, if not identical, results were obtained with purified MT-MMP-1$_{\Delta A}$ (data not shown). Given that none of these substrates were contaminated with detectable quantities of progelatinase A (see "Experimental Procedures"), we conclude that ΔMT-MMP-1 mutants can express intrinsic matrix-degrading activities.

DISCUSSION

Sequence alignments of the 13 human MMPs that have been characterized to date indicate that amino acids 1–508 of the 538-residue-long extracellular domain of MT-MMP-1 contain all of the major structural elements of the secreted members of this gene family (*i.e.* a propeptide and catalytic domain as well as a hemopexin-like region that is bounded by a pair of highly conserved cysteinyl residues; Refs. 4–7). Given that the C termini of virtually all secreted MMPs end at, or extend no more than 8 amino acids beyond, the final cysteinyl residue in the hemopexin domain (7), we reasoned that TM-deletion mutants of MT-MMP-1 that retained this modular organization would encode functional proteinases. Indeed, as demonstrated, regardless of whether soluble mutants of MT-MMP-1 were truncated either at the edge of the TM domain or at the end of the hemopexin domain, the expressed proteins displayed similar, if not identical, activities as assessed by zymography, progelatinase A processing, or substrate specificity.

By itself, our work does not rule out the possibility that the TM or cytosolic domains of MT-MMP-1 convey additional structural information to the processed proteinase (*i.e.* beyond acting as a membrane anchor). However, while our work was in progress, Cao *et al.* (9) reported that an MT-MMP-1 chimera

generated by exchanging the TM and cytosolic domains of the metalloproteinase with those of the IL-2 receptor functioned normally in terms of its ability to mediate progelatinase A activation (9). Although this result is consistent with our conclusion that the extracellular domain of MT-MMP-1 confers the proteinase with its distinct characteristics, these authors also reported that a TM-deletion mutant encoding residues 1–535 of MT-MMP-1 was unable to process progelatinase A (9). In comparing our experimental approaches, it is important to note that the soluble MT-MMP-1 generated in their study was not isolated nor were its interactions with progelatinase A examined directly (9). Instead, Cao *et al.* (9) judged their TM-deletion mutant to be inactive on the basis of its inability to process endogenously derived progelatinase A secreted by COS-1 cells in a transient transfection assay system (9). Under these conditions, however, attempts to assess the activity of secreted MT-MMP-1 would be complicated by the presence of cell-derived TIMPs which can interfere with progelatinase A processing by either inhibiting ΔMT-MMP-1 activity directly, or, in the case of TIMP-2, by binding to the C-terminal domain of the gelatinase zymogen (4, 27, 28). Indeed, when endogenous levels of TIMP are overwhelmed by co-transfecting COS cells with MT-MMP-1$_{\Delta A/B}$ *and* progelatinase A, gelatinase activation can be readily detected in the intact cell system as well as our purified system.[3] Thus, while anchoring a proteinase to the cell membrane might be predicted to more effectively shield an active proteinase from soluble inhibitors (and to perhaps provide a surface more conducive for accelerating processing events) (*e.g.* Refs. 29–31), our data demonstrate that the TM-deletion mutants retain the key functional properties of the wild-type enzyme.

As a consequence of our attempts to characterize the activity of ΔMT-MMP-1, we also discovered that the TM-deletion mutants are capable of undergoing rapid processing to their mature forms. This finding is noteworthy since, as a general rule, MMPs are synthesized and secreted as inactive zymogens (1–3, 7). However, we recently reported that in a fashion similar to that observed for the ΔMT-MMP-1 mutants, prostromelysin-3 is secreted as an active enzyme following its intracellular processing within the constituitive secretory pathway (8). In this case, activation was dependent upon a decapeptide insert that is sandwiched between the pro- and catalytic domains of stromelysin-3 and encrypted with an extended furin recognition motif (*i.e.* RXRXKR). At the time that these earlier studies were completed, stromelysin-3 was the only member of the MMPs family known to contain this recognition sequence. However, with the recent cloning of MT-MMP-1, -2, and -3, it is clear that all three of these enzymes contain homologous inserts which include an array of basic residues (*i.e.* RRKR) that match the recognition motif of the proprotein convertases (*i.e.* RX(K/R)-R; Refs. 4–6).[4] Consistent with this prediction, (i) the N terminus of ΔMT-MMP-1 was located at Tyr[112] on the C-terminal side of the Arg-Arg-Lys-Arg motif, (ii) ΔMT-MMP-1 processing could be inhibited by either inserting point mutations in the RRKR motif or by co-transfecting cells with the furin-specific inhibitor, a_1PI$_{Pitt}$, and (iii) the ΔMT-MMP-1 zymogen could be processed to its active form *ex situ* by soluble furin. While we have not yet identified the intracellular/extracellular compartments in which the ΔMT-MMP-1 zymogen undergoes processing in the intact cell, furin is a membrane-associated endoprotease that not only cycles between the *trans*-

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[4] Although stromelysin-3 and MT-MMP-1 both contain proprotein convertase recognition motifs, comparisons of their genomic organization and chromosomal localization indicate that the two metalloproteinases are not closely related and belong to separate branches of the phylogenetic tree (D. Pei and S. J. Weiss, unpublished observation).

Golgi network and the cell surface, but also undergoes processing to a soluble form that accumulates extracellularly (32–34). Indeed, the possibility that ΔMT-MMP-1 may undergo extracellular processing is further supported by our results with the TM-deleted form of soluble furin. Nonetheless, in spite of the fact that furin is the most credible MT-MMP-1 activator identified to date, caution should be exercised in terms of extrapolating processing pathways that are operative for ΔMT-MMP-1 to the wild-type enzyme. Indeed, in contrast to the results obtained with ΔMT-MMP-1, we and others have found that COS cells transfected with wild-type MT-MMP-1 route most of the enzyme to the cell surface as the unprocessed zymogen rather than the mature enzyme (4, 9, 31).[3,5] Utilizing chimeric constructs between stromelysin-3 and wild-type MT-MMP-1, it appears that while the furin recognition motif in either of the secreted metalloproteinases can be processed effectively, the TM domain of MT-MMP-1 appears to "shield" the recipient proteinase from undergoing rapid intracellular processing.[3] The mechanisms responsible for controlling the intracellular and extracellular processing of wild-type MT-MMP-1 require further analysis, but the fact that the active form of the full-length (10) and mutant enzyme display an identical N terminus directly downstream of the proprotein convertase-recognition motif strongly suggests a role for furin or, perhaps, a related proprotein convertase (*e.g.* PC6; Refs. 24 and 35) in zymogen activation.

In the presence of purified active ΔMT-MMP-1 (but not its zymogen), progelatinase A was processed to its mature form (*i.e.* [Tyr^{81}]gelatinase) via the formation of the Leu^{38} intermediate. This two-step, TIMP-2-sensitive activation cascade is identical with that previously established for crude preparations of plasma membrane-associated MT-MMP-1 (27) and allows us to conclude that ΔMT-MMP-1 can initiate the processing event independently of additional co-factors or substrates. Interestingly, the ability of ΔMT-MMP-1 to directly activate progelatinase A under cell-free conditions contrasts with a recent report by Strongin *et al.* (10) wherein an MT-MMP-1·TIMP-2 complex (rather than MT-MMP-1 alone) was proposed to function as the membrane-associated activator of the gelatinase zymogen. We were unable to reproduce this finding with MT-MMP-1$_{\Delta A/B}$, but cannot rule out the possibility that TIMP-2 plays a more complex role on the membrane surface. However, an interpretation of the data presented by Strongin *et al.* (10) is complicated by the fact that even in the apparent absence of TIMP-2, MT-MMP-1 continued to process progelatinase A to the [Leu^{38}]gelatinase intermediate, but not the final mature form. Thus, it remains possible that TIMP-2 exerts its stimulatory effect by accelerating the inter- or intramolecular autocatalytic conversion of [Leu^{38}]gelatinase to [Tyr^{81}]gelatinase on the cell surface (31) rather than by stimulating MT-MMP-1 activity directly.

To date, the only function ascribed to MT-MMP-1 has been its ability to activate progelatinase A (4, 9, 10, 13, 27, 28). However, we have demonstrated that purified MT-MMP-1$_{\Delta A/B}$ can also degrade a number of extracellular matrix components. These data indicate that the ability of MT-MMP-1-transfected cells to express a heightened invasive potential may not necessarily be linked to progelatinase A activation alone (4). Although our studies have employed a soluble form of MT-MMP-1, we believe that the modular organization of MT-MMP-1 strengthens the likelihood that the membrane-tethered form displays a similar substrate specificity. Furthermore, the potential physiologic relevance of ΔMT-MMP-1 mutants have

been heightened by recent findings which suggest that soluble forms of MT-MMP-1 may be generated *in vivo* (13). Thus, while the MT-MMP-1 antigen has been immunodetected on the surface of cancer cells *in vivo* (4), RNA *in situ* hybridization studies have more recently demonstrated that MT-MMP-1 transcripts are confined to the surrounding stromal cells (13). Should MT-MMP-1 undergo solubilization and intercellular transfer *in situ* (4, 13), tumor cells could potentially use the stroma-derived enzyme to assemble a multicatalytic complex on their surface that would not only arm them with the ability to catalyze progelatinase A activation, but also to express an additional repertoire of proteolytic activities. Additional studies will be required to directly compare the soluble and membrane-anchored forms of MT-MMP-1, but the established catalytic activity of the TM-deletion mutants should provide a useful tool for characterizing the enzymic properties of this new family of membrane-anchored MMPs.

REFERENCES

1. Woessner, J. F., Jr. (1991) *FASEB J.* **5,** 2145–21549
2. Matrisian, L. M. (1992) *BioEssays* **14,** 455–463
3. Stetler-Stevenson, W. G., Aznavoorian, S., and Liotta, L. A. (1993) *Annu. Rev. Cell Biol.* **9,** 541–573
4. Sato, H., Takino, T., Okada, Y., Cao, J., Shinagawa, A., Yamamoto, E., and Seiki, M. (1994) *Nature* **370,** 61–65
5. Will, H., and Hinzmann, B. (1995) *Eur. J. Biochem.* **231,** 602–608
6. Takino, T., Sato, H., Shinagawa, A., and Seiki, M. (1995) *J. Biol. Chem.* **270,** 23013–23020
7. Birkedal-Hansen, H., Moore, W. G. I., Bodden, M. K., Windsor, L. J., Birkedal-Hansen, B., DeCarlo, A., and Engler, J. A. (1993) *Crit. Rev. Oral Biol. Med.* **4,** 197–250
8. Pei, D., and Weiss, S. J. (1995) *Nature* **375,** 244–247
9. Cao, J., Sato, H., Takino, T., and Seiki, M. (1995) *J. Biol. Chem.* **270,** 801–805
10. Strongin, A. Y., Collier, I., Bannikov, G., Marmer, B. L., Grants, G. A., and Goldberg, G. I. (1995) *J. Biol. Chem.* **270,** 5331–5338
11. Brown, P. D., Bloxidge, R. E., Anderson, E., and Howell, A. (1993) *Clin. Exp. Metastasis* **11,** 183–189
12. Brown, P. D., Bloxidge, R. E., Stuart, N. S. A., Gatter, K. C., and Carmichael, J. (1993) *J. Natl. Cancer Inst.* **85,** 574–578
13. Okada, A., Bellocq, J.-P., Rouyer, N. Chenard, M.-P., Rio, M.-C., Chambon, P., and Basset, P. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 2730–2734
14. Tournier, J. M., Polette, M., Hinnrasky, J., Beck, J., Werb, Z., and Basbaum, C. (1994) *J. Biol. Chem.* **269,** 25454–25464
15. Aimes, R. T., and Quigley, J. P. (1995) *J. Biol. Chem.* **270,** 5872–5876
16. Vassalli, J.-D., and Pepper, M. S. (1994) *Nature* **370,** 14–15
17. Brenner, C., and Fuller, R. S. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 922–926
18. Bravo, D. A., Gleason, J. B., Sanchez, R. I., Roth, R. A., and Fuller, R. S. (1994) *J. Biol. Chem.* **269,** 25830–25837
19. de Vries, T., Srnka, C. A., Palcic, M. M., Swiedler, S. J., van den Eijnden, D. H., and Macher, B. A. (1995) *J. Biol. Chem.* **270,** 8712–8722
20. Pei, D., Majmuder, G., and Weiss, S. J. (1994) *J. Biol. Chem.* **269,** 25849–25855
21. Davies, B., Brown, P. Q., East, N., Crimmin, M. J., and Balkwill, E. R. (1993) *Cancer Res.* **53,** 2087–2091
22. Fridman, R., Fuerst, T. R., Bird, R. E., Hoyhtya, M., Oelkuct, M., Kraus, S., Komarek, D., Liotta, L., Berman, M. L., and Stetler-Stevenson, W. G. (1992) *J. Biol. Chem.* **267,** 15398–15405
23. Watanabe, T., Murakami, K., and Nakayama, K. (1993) *FEBS Lett.* **320,** 215–218
24. Seidah, N. G., Chretien, M., and Day, R. (1994) *Biochimie (Paris)* **76,** 197–209
25. Rehemtulla, A., and Kaufman, R. J. (1992) *Blood* **79,** 2349–2355
26. Wasley, C. L., Rehemtulla, A., Bristol, J. A., and Kaufman, R. J. (1993) *J. Biol. Chem.* **268,** 8458–8465
27. Strongin, A. Y., Marmer, B. L., Grant, G. A., and Goldberg, G. I. (1993) *J. Biol. Chem.* **268,** 14033–14039
28. Ward, R. V., Atkinson, S. J., Reynolds, J. J., and Murphy, G. (1994) *Biochem. J.* **304,** 263–269
29. Bangalore, N., and Travis, J. (1994) *Biol. Chem. Hoppe-Seyler.* **375,** 659–666
30. Young, T. N., Pizzo, S. V., and Stack, M. S. (1995) *J. Biol. Chem.* **270,** 999–1002
31. Atkinson, S. J., Crabbe, T., Cowell, S., Ward, R. V., Butler, M. J., Sato, H., Seiki, M., Reynolds, J. J., and Murphy, G. (1995) *J. Biol. Chem.* **270,** 30479–30485
32. Vidricaire, G., Denault, J.-B., and Leduc, R. (1993) *Biochem. Biophys. Res. Commun.* **195,** 1011–1018
33. Molloy, S. S., Thomas, L., VanSlyke, J. K., Stenberg, P. E., and Thomas, G. (1994) *EMBO J.* **13,** 18–33
34. Vey, M., Schafer, W., Berghofer, B., Klenk, H.-D., and Garten, W. (1994) *J. Cell Biol.* **127,** 1829–1842
35. Horimoto, T., Nakayama, K., Smeekens, S. P., and Kawaoka, Y. (1994) *J. Virol.* **68,** 6074–6078

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[5] However, a portion of the MT-MMP-1 zymogen does undergo processing since the mature enzyme has been isolated from HT-1080 plasma membranes (10) and is required for progelatinase activation.